ARTICLE
Journal of Cellular Biochemistry 110:1147–1154 (2010)

Journal of Cellular Biochemistry

Molecular Silencing of Twist1 Enhances Osteogenic Differentiation of Murine Mesenchymal Stem Cells: Implication of FGFR2 Signaling

Hichem Miraoui,[1] Nicolas Severe,[1] Pascal Vaudin,[2] Jean-Christophe Pagès,[2] and Pierre J. Marie[1*]

[1]*Laboratory of Osteoblast Biology and Pathology, Inserm U606, University Paris Diderot, Hôpital Lariboisiere, Paris, France*

[2]*Inserm U966, University of Tours, Tours, France*

ABSTRACT

The capacity of mesenchymal stem cells (MSCs) to differentiate into functional osteoblasts is tightly controlled by transcription factors that trigger osteoblast commitment and differentiation. The role of Twist1, a basic helix-loop-helix (bHLH) transcription factor, in osteogenic differentiation of MSCs remains unclear. Here we investigated the role of Twist1 in the osteogenic differentiation program of murine C3H10T1/2 mesenchymal cells. We showed that molecular silencing of Twist1 using short hairpin RNA (shRNA) expression moderately increased C3H10T1/2 cell proliferation and had no effect on cell survival. In contrast, Twist1 silencing enhanced osteoblast gene expression and matrix mineralization in vitro. Biochemical analyses revealed that Twist1 silencing increased the expression of FGFR2 protein level, which was reduced by a mutant Runx2. Consistent with this finding, Twist1 silencing increased ERK1/2 and PI3K signaling. Moreover, molecular or pharmacological inhibition of FGFR2 or of ERK1/2 and PI3K signaling partly abolished the increased osteoblast gene expression induced by Twist1 silencing in C3H10T1/2 cells. These results reveal that Twist1 silencing upregulates osteoblast differentiation of murine mesenchymal cells in part via activation of FGFR2 expression and downstream signaling pathways, which provides novel insights into the molecular signals by which this transcription factor regulates the osteogenic differentiation program in MSCs. J. Cell. Biochem. 110: 1147–1154, 2010.

KEY WORDS: TWIST1; OSTEOGENIC DIFFERENTIATION; MESENCHYMAL STEM CELLS; FGF RECEPTOR SIGNALING

Mesenchymal stem cells (MSCs) have the capacity to differentiate into different cell types including osteogenic cells under appropriate stimulation by hormonal and local factors [Pittenger et al., 1999; Bianco et al. 2001; Marie and Fromigué, 2006]. In vitro, the osteogenic differentiation of MSCs is characterized by the early expression of the master osteoblast transcription factor Runx2, followed by the expression of alkaline phosphatase (ALP), type I collagen (Col1A1), and extracellular matrix mineralization [Lian et al., 2004]. The capacity of MSCs to differentiate into functional osteoblasts is controlled by transcription factors that trigger osteoblast commitment and differentiation in a tightly regulated manner [Lian et al., 2004; Franceschi et al. 2007; Marie, 2008].

Twist is a basic helix-loop-helix (bHLH) transcription factor involved in mesodormal and myoblast differentiation [Chen and Behringer, 1995]. Twist heterodimerizes with the broadly expressed bHLH E proteins that bind DNA canonical sequences called E-boxes (CANNTG) which are consensus binding sites for bHLH proteins present in the promoter of target genes [Castanon and Baylies, 2002]. Twist1 is expressed in the skeletal mesenchyme and plays an important role in the control of cranial suture development [Johnson et al., 2000; Rice et al., 2000]. Notably, genetic mutations in the

Grant sponsor: Inserm Programme National de Recherche (Pro-A Inserm, France); Grant sponsor: la Fondation de l'Avenir (Paris, France); Grant number: ER6-417; Grant sponsor: Association Rhumatisme & Travail (Hôpital Lariboisière, Paris, France).

Pascal Vaudin's present address is UMR Physiologie de la Reproduction et des Comportements, INRA Centre de Tours, 37380 Nouzilly, France.

*Correspondence to: Dr. Pierre J. Marie, Inserm U 606, University Paris Diderot, Hôpital Lariboisiere, 2 rue Ambroise Pare, 75475 Paris Cedex 10, France. E-mail: pierre.marie@inserm.fr

Received 24 November 2009; Accepted 24 March 2010 • DOI 10.1002/jcb.22628 • © 2010 Wiley-Liss, Inc.
Published online 17 May 2010 in Wiley InterScience (www.interscience.wiley.com).

Twist1 gene in the Saethre–Chotzen syndrome cause Twist1 haploinsufficiency and premature cranial suture fusion [El Ghouzzi et al., 1997; Howard et al., 1997] as a result of increased bone formation [Yousfi et al., 2001], indicating that Twist1 is an important transcription factor controling osteoblastogenesis [Rice et al., 2000; Marie et al., 2008]. Previous studies showed that Twist1 controls cell proliferation, differentiation, and survival in osteoblastic cells [Lee et al., 1999; Yousfi et al., 2001, 2002a; Bialek et al., 2004; Guenou et al., 2006; Komaki et al., 2007; Zhang et al., 2008]. Notably, Twist1 was found to control osteoprogenitor cell differentiation via modulation of Runx2 [Bialek et al., 2004; Zhang et al., 2008; Yousfi et al., 2002b; Guenou et al., 2005] and fibroblast growth factor (FGF) [Rice et al., 2000; O'Rourke et al., 2002; Guenou et al., 2005; Connerney et al., 2008]. However, the mechanisms by which Twist1 may control the early stages of differentiation in MSCs remain unclear.

Based on our previous demonstration that Twist1 haploinsufficiency enhances cranial bone formation in the Saethre–Chotzen syndrome [Yousfi et al., 2001], we hypothesized that Twist1 silencing may have a positive effect on the commitment and differentiation of osteoblast precursor cells. We therefore investigated the effect of Twist1 silencing on MSC osteogenic differentiation and determined the mechanisms involved in the control of Twist1 silencing on osteoblast commitment and differentiation.

MATERIALS AND METHODS

CELLS AND REAGENTS

Murine pluripotent mesenchymal C3H10T1/2 cells were obtained from the ATCC (Rockville, MD). We also used human clonal pluripotent bone marrow stromal Stro-1+ cells obtained as previously described [Oyajobi et al., 1999]. Cells were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen Corporation, Paisley, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine and penicillin/streptomycin (10,000 U/ml and 10,000 μg/ml, respectively), at 37°C in humidified atmosphere containing 5% CO_2 in air. Culture media were changed every 2 days. Anti-FGFR2, anti-phospho-tyrosine (p-Tyr), anti-ERK, and anti-p-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies (c-Cbl, anti-PI3K p85, anti-p-PI3K p85, and anti-Snail (pan antibody) were from Cell Signaling (Sigma–Aldrich, Saint-Quentin Fallavier, France). The β-actin antibody, U0126 (an inhibitor of MAPK kinase 1 and 2 (MEK1/2) that blocks phosphorylation and activation of ERK1/2 and the PI3K inhibitor wortmannin were from Sigma (St. Louis, USA).

EXPRESSION VECTORS

For the plasmid pLenti-shTwist1 construction, shTwist1 encoding sequence was obtained by PCR elongation of the primer shTwist1-*Bam*HI 5′-GGATCCCCAAGCTGAGCAAGATTCAGACCTTCAAGA-GAGGTC-3′ annealed to a second primer shTwist1-*Hin*dIII 5′-AA-GCTTAAAAAAAGCTGAGCAAGATTCAGACCTCTCTTGAAG-3′.

The shTwist1 encoding sequence was cloned into pGEMT easy vector (Promega) and transferred into the pH1 plasmid (gift from Anne Galy, Généthon-Evry, France) at *Bgl*II/*Hin*dIII sites. The H1 promoter-shTwist1 sequence was then cloned into the pLenti-RNAi vector at *Spe*I and *CLa*I sites. For lentiviral particle production, 293T cells were plated at 125,000 cells/cm^2 in culture Petri dishes and grown overnight at 37°C. The culture medium was replaced 3 h before transfection. Transfection was performed with plasmids ph-CMV-G, p8.74 (a gift from D. Trono) and pLenti-sh-Twist1 using a calcium phosphate transfection kit (Invitrogen) according to the manufacturer's instructions. Supernatant was harvested 48 and 72 h later, filtered through a 0.45 μm Millex HA filter (Millipore Corporation, Bedford, USA) and lentiviral vectors were concentrated on a 20% sucrose cushion by centrifugation at 26k rpm during 90 min. Pellets were left for resuspension overnight at 4°C in PBS. After gentle pipeting, aliquots were stored at −80°C. The viral titer was determined by GFP cell counting using a FACS analysis of transduced 293T cells and expressed transducing unit per milliliters (TU/ml).

To analyze the respective role of FGFR2, Runx2, and ERK1/2 in osteoblast differentiation induced by Twist1 silencing, C3H10T1/2 cells were transiently transfected with FGFR2 siRNA or scramble RNA (kindly provided by Dr. F. Jakob, University of Wurzburg, Germany), a Runx2 mutant cDNA which bears a Ser-191Asn mutation in the runt domain that impairs DNA binding kindly provided by Dr. G. Karsenty (Columbia University, New York, NY, USA) [Lee et al., 1997], a dominant-negative ERKp44-MAPK kinase-deficient mutant vector (DN-ERK) [Pagès et al., 1993] or empty vectors and Exgen (Euromedex) according to the manufacturer's instructions.

CELL PROLIFERATION AND APOPTOSIS, ALP ACTIVITY AND IN VITRO OSTEOGENESIS

For cell proliferation assay, infected C3H10T1/2 cells were cultured in six-well plates (10^5 cells per well) and cell number was evaluated by cell counting. For determination of cell apoptosis, cells were cultured in the presence (10%) or absence of FCS for 24 h and apoptosis was determined by the Apopercentage assay [Miraoui et al., 2009]. For ALP staining, we used the Sigma FAST kit according to the manufacturer's recommendations (Sigma). Briefly, cells were fixed in 75% ethanol, rinsed in PBS and incubated with the substrate buffer at 37°C. For in vitro matrix mineralization, cell culture medium was supplemented with 50 μmol/L ascorbic acid and 3 mM inorganic phosphate (NaH_2PO_4) to allow matrix synthesis and mineralization. At the indicated time points, cells were fixed in 4% paraformaldehyde in PBS. Matrix mineralization was evaluated by alizarin red staining as described [Miraoui et al., 2009] and photographed using an Olympus microsocope (Japan).

QUANTITATIVE RT-PCR ANALYSIS

Total RNAs were isolated using Trizol reagent (InVitrogen, France). Three micrograms of total RNA from each samples were reverse transcribed using MMLV reverse transcriptase and oligodT primers at 37°C for 90 min. Relative mRNA levels were evaluated by quantitative PCR (LightCycler; Roche Applied Science, Indianapolis, OH) using a SYBR Green PCR kit (ABGen, Courtabœuf, France) and

specific primers as described [Miraoui et al., 2009]. Signals were normalized to GAPDH as internal control.

WESTERN BLOT ANALYSIS

Cell lysates were prepared as described [Miraoui et al., 2009]. Briefly, proteins (30 μg) were resolved on 4–12% SDS–PAGE and transferred onto PVDF nitrocellulose membranes (Millipore Corporation). Filters were incubated for 2 h in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1% (v/v) Tween-20, 0.5% (w/v) bovine serum albumin (TBST/BSA), then overnight at 4°C on a shaker with specific primary antibodies (1/500–1/1,000 in TBST/BSA). Membranes were washed twice with TBST and incubated for 2 h with appropriate HRP-conjugated secondary antibody (1/10,000–1/20,000 in TBST/BSA). After final washes, the signals were visualized with enhanced chemiluminescence Western blotting detection reagent (ECL, Amersham Biosciences, Piscataway, NJ) and autoradiographic film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY). Densitometric analysis using ImageQuant software was performed following digital scanning (Agfa). Representative images of immunoblots are shown.

STATISTICAL ANALYSIS

The data are the mean ± standard deviation (SD) of an average of six samples and are representative of at least two distinct experiments. The data were analyzed by Student's *t*-test and a minimal level of $P < 0.05$ was considered significant.

RESULTS

EFFECT OF TWIST1 SILENCING ON MSC PROLIFERATION AND SURVIVAL

We first checked that shTwist1 was efficient in decreasing Twist protein expression in C3H10T1/2 mesenchymal cells. As shown in Figure 1A, shTwist1 infection reduced Twist1 expression in C3H10T1/2 cells. Quantitative analysis of the blot showed that shTwist1 reduced Twist1 mRNA expression by about 50% (Fig. 1B), validating the efficiency of Twist silencing in these cells. We then investigated the effects of Twist1 silencing on cell proliferation. A shown in Figure 1C, Twist1 silencing modestly increased cell number. In contrast, shTwist1 had no effect on cell survival even in pro-apoptotic conditions (serum-deprived medium) (Fig. 1D). These results indicate that a 50% decrease in Twist1 moderately enhances cell proliferation but has no effect on cell survival in C3H10T1/2 cells.

TWIST1 SILENCING PROMOTES MSC OSTEOBLAST DIFFERENTIATION

We next determined the effect of Twist1 silencing on the osteogenic differentiation program in C3H10T1/2 cells. As shown in Figure 2A, Twist1 silencing slightly increased Runx2 and osteocalcin expression, and had a more positive effect on ALP and COL1A1 expression, suggesting that Twist1 silencing promotes the expression of genes involved in osteoblast differentiation of MSCs. To confirm that Twist1 silencing enhances the osteogenic

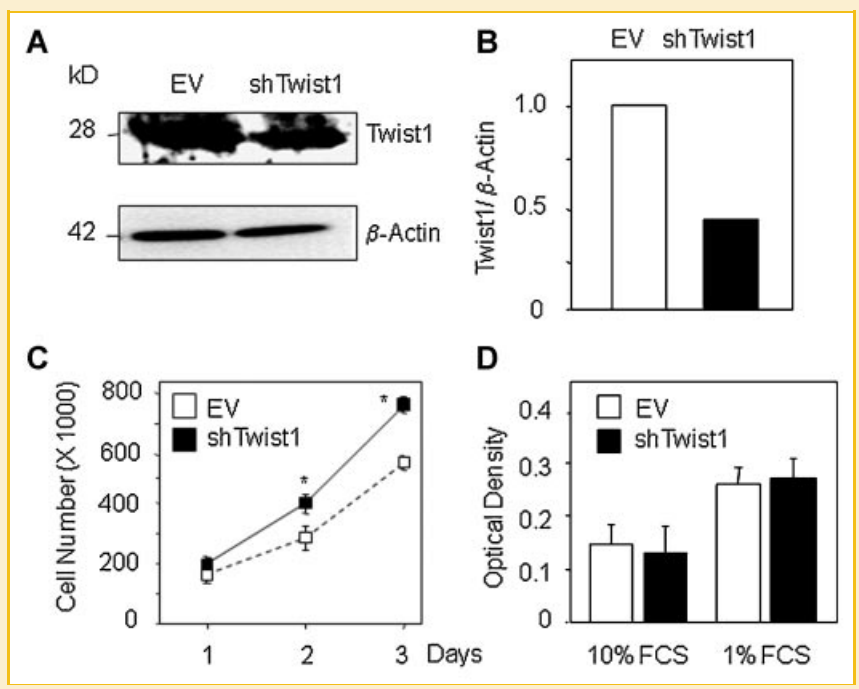

Fig. 1. Twist1 silencing promotes mesenchymal cell proliferation but not survival. Western blot analysis (A) and quantification analysis (B) showing decreased Twist1 protein levels in C3H10T1/2 cells infected with shTwist1 compared to the empty vector (EV). Time course analysis showed that shTwist1 moderately increased cell number compared to control cells infected with EV (C). shTwist1 had no effect on cell survival determined by the Apopercentage assay in the presence of 10% serum or in serum-deprived (1%) conditions at 24 h (D). Data are the mean ± SD. Asterisk (*) indicates a significant difference with EV-transfected cells ($P < 0.05$).

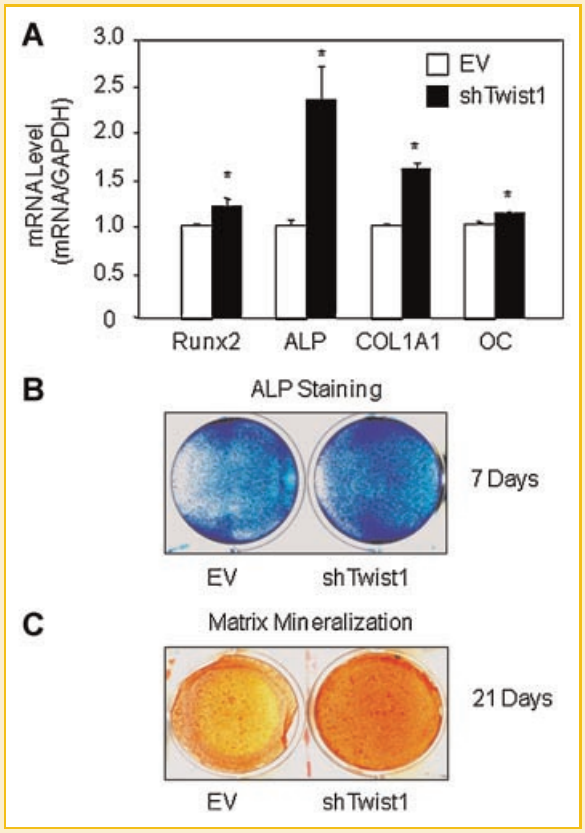

Fig. 2. Twist1 silencing promotes osteogenic differentiation in mesenchymal cells. Quantitative PCR analysis showing that shTwist1 increased the expression of osteoblast markers in C3H10T1/2 cells compared to empty vector (EV)-infected cells (A). Functional analysis showing that shTwist increased ALP staining (B) and matrix mineralization compared to EV (C). Data are the mean ± SD. Asterisk (*) indicates a significant difference with EV-transfected cells ($P < 0.05$).

differentiation program in these MSCs, we determined its effect on ALP staining and in vitro osteogenesis in long-term culture. As shown in Figure 2B,C, shTwist1 clearly increased ALP staining and matrix mineralization in MSCs. These results establish that Twist1 silencing enhances osteoblast differentiation and osteogenic capacity in cultured MSCs.

TWIST1 SILENCING INCREASES FGFR2 EXPRESSION AND SIGNALING IN MSCs

Having demonstrated that Twist1 silencing results in increased osteogenic differentiation program in MSCs, we investigated the mechanisms by which Twist1 upregulates osteoblast differentiation. We previously reported that Twist1 modulates FGFR2 expression in mature human osteoblasts [Guenou et al., 2005] and that FGFR2 positively controls osteoblast differentiation in murine MSCs [Miraoui et al., 2009]. We thus postulated that the increased osteoblast differentiation induced by Twist1 silencing may result, at least in part, from change in FGFR2 expression. As shown in Figure 3A, Western blot analysis revealed that shTwist1 increased FGFR2 protein level in C3H10T1/2 cells. Quantification analysis showed that FGFR2 expression was upregulated by twofold by Twist1 silencing (Fig. 3B). These results suggest that Twist silencing may promote osteogenic differentiation in C3H10T1/2 cells indirectly at least in part via FGFR2 expression.

One possible mechanism by which receptor tyrosine kinases (RTKs) such as FGFR2 are regulated is via alteration of its degradation by the proteasome [Dikic and Giordano, 2003]. Cbl is an important ubiquitin ligase that controls ubiquitination and degradation of RTKs [Thien and Langdon, 2001; Swaminathan and Tsygankov, 2006], including FGFR2 [Kaabeche et al., 2004]. We previously showed that Twist1 haploinsufficiency downregulates Cbl expression in mature osteoblasts [Guenou et al., 2006]. We therefore investigated whether Twist silencing may affect Cbl expression in MSCs. As shown in Figure 3C and confirmed in Figure 3D, Cbl protein level was unchanged by shTwist1 in C3H10T1/2 mesenchymal cells, suggesting that the increased FGFR2 expression induced by Twist1 silencing was not related to alteration in Cbl-mediated ubiquitination in these cells. Interestingly, the transcription factors Twist and Snail are downstream targets of FGF signaling [Rice et al., 2005] and mutations in Snail and Twist play an important role in pathological cranial suture formation in the mouse [Oram and Gridley, 2005]. We therefore analyzed potential relationships between Twist and Snail in C3H10T1/2 cells. As shown in Figure 3D, Snail protein level was not affected by Twist1 silencing despite increased FGFR2 signaling, suggesting that the increased osteoblast gene expression induced by Twist1 silencing was not related to Snail expression in these cells.

ACTIVATED FGFR2 SIGNALING MEDIATES OSTEOBLAST DIFFERENTIATION INDUCED BY TWIST1 SILENCING IN MSCs

Several signaling pathways are known to be activated by FGFR2 [Eswarakumar et al., 2005]. Recently, ERK1/2 and PI3K signaling pathways have been reported to play an important role in osteoblastogenesis [Jaiswal et al., 2000; Almeida et al., 2005; Kawamura et al., 2007; Dufour et al., 2008]. We therefore investigated whether the increased FGFR2 expression induced by Twist1 silencing translated into changes in ERK1/2 and PI3K signaling. We found that the increased FGFR2 expression induced by shTwist1 was associated with a twofold increase in ERK1/2 phosphorylation (Fig. 3E,F). Additionally, shTwist1 increased PI3K phosphorylation in C3H10T1/2 cells (Fig. 3G,H). These results show that the increased FGFR2 expression induced by shTwist1 in MSCs results in increased ERK1/2 and PI3K signaling. We then determined whether the increased ERK1/2 and PI3K signaling may be involved in MSC osteoblast differentiation induced by Twist1 silencing. To determine the role of ERK1/2 and PI3K on osteoblast differentiation induced by Twist silencing in C3H10T1/2 cells, cells infected with shTwist1 were transfected with DN-ERK and the expression of osteoblast differentiation markers was determined. As shown in Figure 4A, OC expression was decreased in shTwist1-infected cells transfected with DN-ERK1/2 compared to control cells transfected with the empty vector. Similarly, pharmacological inhibition of PI3K decreased COL1A1 and OC expression in these cells (Fig. 4B). These results indicate that ERK1/2 and PI3K activation signaling contribute, at least in part, to the increased osteoblast differentiation induced by Twist1 silencing in C3H10T1/2 cells.

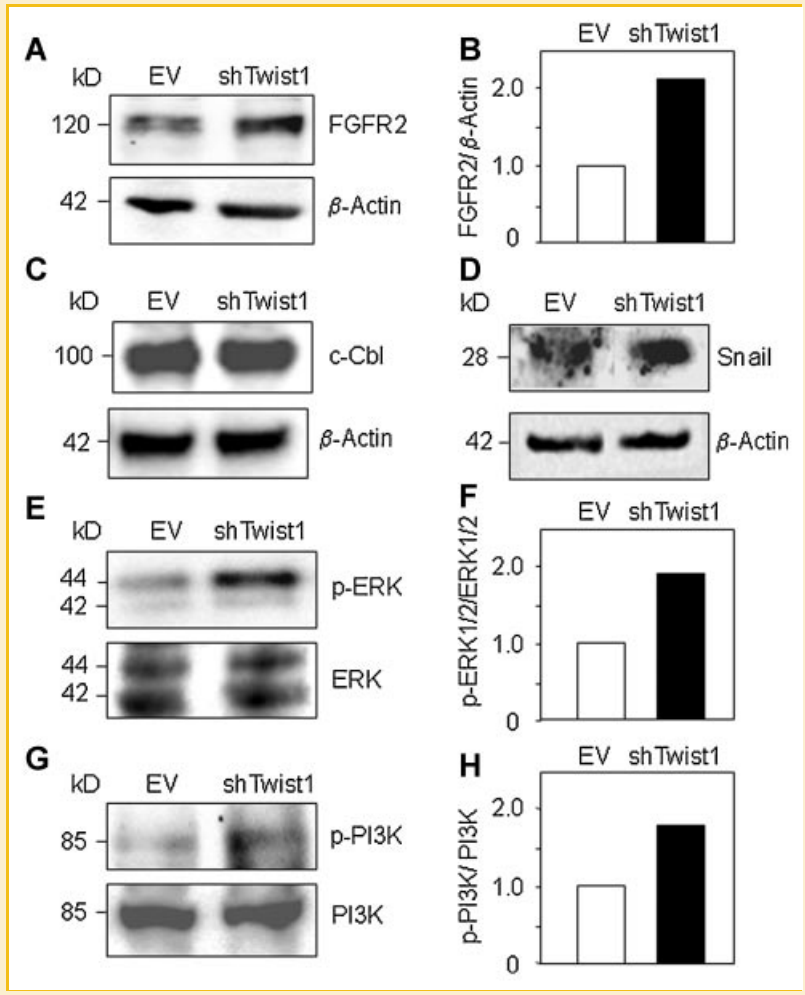

Fig. 3. Role of FGFR2 signaling in osteoblast differentiation induced by shTwist1 in mesenchymal cells. Western blot analysis (A) and quantification of blots (B) showing increased FGFR2 levels in shTwist1-infected cells compared to cells infected with empty vector (EV). Western blot analysis showed that Cbl (C) and Snail (D) levels were unchanged by shTwist1. Western blot analysis and quantification of blots showed that Twist1 silencing increased phosphorylated (p)-ERK1/2 (E,F) and p-PI3K levels (G,H) compared to empty vector (EV) in C3H10T1/2 cells. β-Actin was used as loading control.

To confirm the implication of FGFR2 in the effects of Twist1 silencing in MSCs, shTwist1-infected cells were transfected with a FGFR2 or scramble siRNA and osteoblastic genes were determined. As shown in Figure 5A, siFGFR2 decreased Runx2, ALP, and Col1A1 mRNA levels but had no effect on OC mRNA levels, indicating that FGFR2 mediates, at least in part, the induction of these genes by Twist1 silencing in MSCs. Our previous studies indicate that Runx2 controls FGFR2 promoter activity and mRNA expression in osteoblasts [Guenou et al., 2005]. It is thus possible that the observed increase in Runx2 level induced by Twist1 silencing may contribute to increase FGFR2 expression in MSCs. To analyze whether the increased FGFR2 expression induced by Twist1 silencing may be related to Runx2 activity in MSCs, shTwist1-infected cells were transfected with a Runx2 mutant bearing a Ser-191Asn mutation in the runt domain that impairs DNA binding, or the empty vector [Lee et al., 1997]. We found that transfection with the Runx2 mutant decreased FGFR2 expression induced by shTwist1 (Fig. 5B), indicating that Runx2 mediates, at least in part, FGFR2 expression induced by shTwist1. Overall, these data reveal that Twist1 silencing enhances osteogenic differentiation and in vitro matrix mineralization in murine C3H10T1/2 mesenchymal cells in part via increased Runx2 and FGFR2 expression and subsequent activation of ERK1/2 and PI3K signaling (Fig. 5C).

DISCUSSION

The mechanisms by which the bHLH factor Twist1 controls osteoblastogenesis are not fully understood. Here we report that Twist1 silencing upregulates the osteogenic program in uncommitted MSCs in part via FGFR2 signaling. We first showed that molecular silencing of the Twist1 gene slightly increased C3H10T1/2 cell proliferation in murine mesenchymal cells. These data are consistent with our previous finding that Twist haploinsufficiency leads to expand the osteogenic precursor cell population in human calvaria osteoblasts [Yousfi et al., 2001; Guenou et al., 2005]. In

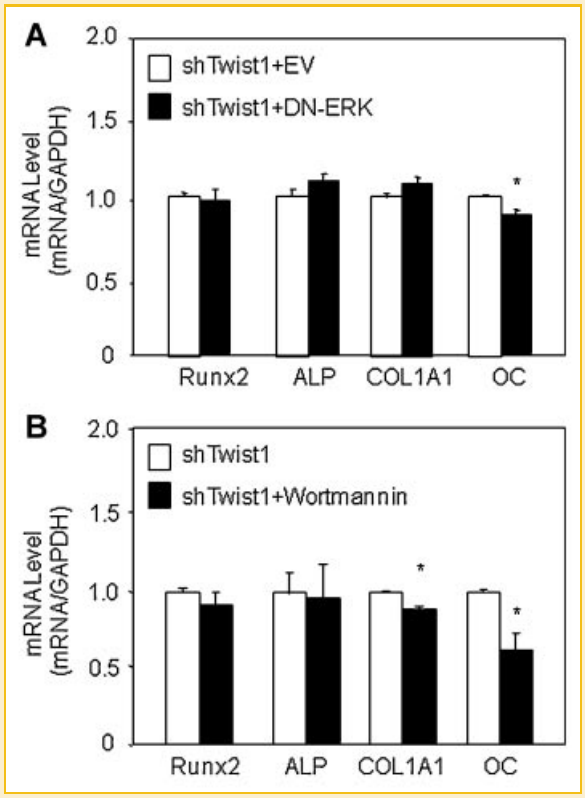

Fig. 4. ERK1/2 and PI3K signaling mediates in part osteoblast differentiation induced by Twist1 silencing. Transient transfection with DN-ERK1/2 (A) or treatment with the PI3K inhibitor wortmannin (B) abolished the expression of phenotypic osteoblast genes (COL1A1, OC) induced by shTwist1 in C3H10T1/2 cells. Data are the mean ± SD. Asterisk (*) indicates a significant difference with control cells treated with empty vector or solvent ($P < 0.05$).

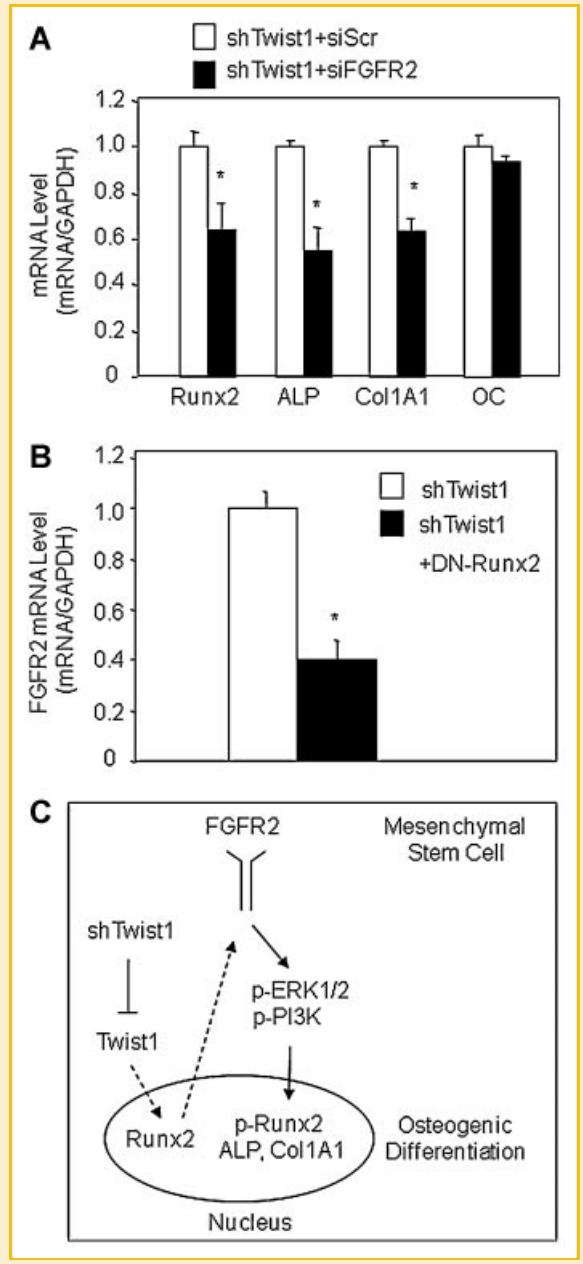

Fig. 5. Runx2 and FGFR2 signaling in part mediates osteoblast differentiation induced by Twist1 silencing. Transient transfection with FGFR2 siRNA (siFGFR2) (A) or DN-Runx2 (B) markedly decreased phenotypic osteoblast gene expression induced by shTwist1 in C3H10T1/2 cells. Data are the mean ± SD. Asterisk (*) indicates a significant difference with control cells treated with empty vector or scramble RNA (siScr) ($P < 0.05$). Proposed mechanisms by which Twist1 silencing enhances the osteogenic differentiation in murine mesenchymal stem cells (C). Twist1 silencing induced by shTwist1 acts in part by increasing Runx2 and FGFR2 expression (dotted lines) which in turn activates ERK1/2 and PI3K signaling that induces osteoblast differentiation in murine C3H10T1/2 mesenchymal stem cells.

contrast to the effect of Twist1 haploinsufficiency in mature osteoblasts [Yousfi et al., 2002a], we found that Twist1 silencing had no effect on MSC survival, revealing a stage-specific control of osteoblast apoptosis by Twist1. Importantly, we found that molecular silencing of the Twist1 gene promoted the osteoblast differentiation program and in vitro osteogenic capacity in murine MSCs. This effect does not appear to be restricted to murine MSCs since we observed similar effects in human clonal bone marrow derived mesenchymal cells [Oyajobi et al., 1999] (data not shown). Consistent with our data, it was recently reported that Twist overexpression inhibits osteoblast differentiation in MSCs [Isenmann et al., 2009]. Our finding that Twist silencing promotes the osteogenic differentiation program in murine MSCs provides one mechanism by which Twist haploinsufficiency caused by Twist1 genetic mutations in the Saethre–Chotzen syndrome enhances the osteogenic capacity of cranial osteoblasts in vitro and in vivo [Yousfi et al., 2001; Guenou et al., 2005].

An important issue was to determine the mechanisms underlying the increased osteogenic differentiation induced by Twist1 silencing in MSCs. Previous studies suggested that Twist proteins may interact with FGF signaling in cranial mouse bone [Rice et al., 2000; Funato et al., 2001; Connerney et al., 2008]. However, the control of FGFR2 expression by Twist is complex [Connerney et al., 2006]. Indeed, Twist1 was found to upregulate or to repress FGFR2 expression in vivo, depending on the balance between Twist1/E-protein heterodimers [Connerney et al., 2008]. In the present study, we show that Twist1 silencing increased FGFR2 expression and signaling in

murine MSCs. This may have functional implications in MSC osteoblast differentiation since FGF signaling plays an important role in osteoblastogenesis [Marie, 2003; Dailey et al., 2005] and we recently showed that activated FGFR2 increases osteoblast differentiation in murine MSCs [Miraoui et al., 2009]. However, we found that shTwist1 cannot further increase osteoblast gene expression in MSCs overexpressing wild-type or activating mutant FGFR2 (data not shown). In addition to the implication of FGFR2, other molecular mechanisms may be involved in osteogenic differentiation of MSCs by Twist1. For example, Twist1 was found to modulate periostin expression and BMP signaling that are potent activators of osteoblast differentiation [Oshima et al., 2002; Hayashi et al., 2007].

One mechanism by which Twist1 may control FGFR2 protein level is alteration of Cbl, a major ubiquitin ligase that negatively controls RTKs [Swaminathan and Tsygankov, 2006]. Cbl negatively controls FGFR protein levels [Kaabeche et al., 2004] and Twist haploinsufficiency in human osteoblasts decreases Cbl-mediated PI3K degradation, causing activation of PI3K signaling [Guenou et al., 2006]. In the present study, Twist1 silencing in murine MSCs had no apparent effect on Cbl protein levels, suggesting that the increased FGFR2 induced by Twist1 silencing at the protein level did not result from decreased FGFR2 protein ubiquitination. Another important effector linked to FGF signaling is Runx2 [Rice et al., 2000; Yousfi et al., 2002b; Bialek et al., 2004; Guenou et al., 2005; Ling et al., 2006; Tang et al., 2007; Teplyuk et al., 2009]. We found that Twist1 silencing increased Runx2 expression in murine MSCs, which is in contrast with the effect of Twist1 haploinsufficiency in human mature osteoblasts [Guenou et al., 2005], thus revealing another cell stage-specific mechanism of control of Runx2 and osteoblast differentiation by Twist1. We showed that inhibition of Runx2 transcriptional activity using a Runx2 mutant abolished FGFR2 gene expression in shTwist1-infected MSCs, indicating that the increased FGFR2 expression induced by Twist1 silencing in MSCs involves Runx2 activity. These data thus provide a novel positive interaction between Twist1, Runx2, and FGFR2 in the control of osteoblast differentiation of MSCs.

A remaining issue was to determine the downstream signaling mechanisms that mediate the positive effect of Twist1 silencing on osteogenic differentiation in MSCs. Multiple signals are known to be activated by FGF/FGFR signaling [Eswarakumar et al., 2005]. Notably, ERK1/2 and PI3K pathways were found to upregulate osteoblast differentiation in MSCs [Jaiswal et al., 2000; Lai et al., 2001; Miraoui et al., 2009]. Our data indicate that FGFR2 activation induced by Twist1 silencing resulted in activation of ERK1/2 and PI3K phosphorylation, suggesting a role for these pathways in the osteoblast phenotype induced by Twist1 silencing in these cells. Consistently, molecular or pharmacological inhibition of FGFR2, ERK1/2, or PI3K activity partially abolished the increased osteoblast gene expression induced by shTwist1. These findings indicate that FGFR2, ERK1/2, and PI3K pathways mediate, at least in part, the positive effect of Twist1 silencing on osteoblast differentiation in murine MSCs (Fig. 5C).

In summary, the present data indicate that Twist1 silencing enhances the osteogenic differentiation program in MSCs and reveal that this anabolic effect results from mechanisms involving upregulation of Runx2 and FGFR2 and downstream signaling pathways. This provides novel insights into the molecular signals by which the transcription factor Twist1 regulates osteogenic differentiation in murine mesenchymal cells.

ACKNOWLEDGMENTS

This work was funded in part by Inserm Programme National de Recherche (Pro-A Inserm, France), la Fondation de l'Avenir (ER6-417, Paris, France) and Association Rhumatisme & Travail (Hôpital Lariboisière, Paris, France). Hichem Miraoui is a recipient of a Ph.D. Award from the Ministère de la Recherche (Paris, France).

REFERENCES

Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. 2005. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signalling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J Biol Chem 280:41342–41351.

Bialek P, Kern B, Yang X, Schrock M, Sosic D, Hong N, Wu H, Yu K, Ornitz DM, Olson EN, Justice MJ, Karsenty G. 2004. A twist code determines the onset of osteoblast differentiation. Dev Cell 6:423–435.

Bianco P, Riminucci M, Gronthos S, Robey PG. 2001. Bone marrow stromal stem cells: Nature, biology, and potential applications. Stem Cells 19: 180–192.

Castanon I, Baylies MK. 2002. A Twist in fate: Evolutionary comparison of Twist structure and function. Gene 287:11–22.

Chen ZF, Behringer RR. 1995. Twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev 9:686–699.

Connerney J, Andreeva V, Leshem Y, Muentener C, Mercado MA, Spicer DB. 2006. Twist1 dimer selection regulates cranial suture patterning and fusion. Dev Dyn 235:1345–1357.

Connerney J, Andreeva V, Leshem Y, Mercado MA, Dowell K, Yang X, Lindner V, Friesel RE, Spicer DB. 2008. Twist1 homodimers enhance FGF responsiveness of the cranial sutures and promote suture closure. Dev Biol 318:323–334.

Dailey L, Ambrosetti D, Mansukhani A, Basilico C. 2005. Mechanisms underlying differential responses to FGF signalling. Cytokine Growth Factor Rev 16:233–247.

Dikic I, Giordano S. 2003. Negative receptor signalling. Curr Opin Cell Biol 15:128–135.

Dufour C, Guenou H, Kaabeche K, Bouvard D, Sanjay A, Marie PJ. 2008. FGFR2-Cbl interaction in lipid rafts triggers attenuation of PI3K/Akt signalling and osteoblast survival. Bone 42:1032–1039.

El Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Gonzales-Ramos M, Kline AD, Jabs EW. 1997. Mutations in Twist, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. Nat Genet 15:36–41.

Eswarakumar VP, Lax I, Schlessinger J. 2005. Cellular signalling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 16:139–149.

Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. 2007. Transcriptional regulation of osteoblasts. Ann NY Acad Sci 1116:196–207.

Funato N, Ohtani K, Ohyama K, Kuroda T, Nakamura M. 2001. Common regulation of growth arrest and differentiation of osteoblasts by helix-loop-helix factors. Mol Cell Biol 21:7416–7428.

Guenou H, Kaabeche K, Le Mee S, Marie PJ. 2005. A role for fibroblast growth factor receptor-2 in the altered osteoblast phenotype induced by Twist haploinsufficiency in the Saethre-Chotzen syndrome. Hum Mol Genet 14: 1429–1439.

Guenou H, Kaabeche K, Dufour C, Miraoui H, Marie PJ. 2006. Down-regulation of ubiquitin ligase Cbl induced by twist haploinsufficiency in Saethre-Chotzen syndrome results in increased PI3K/Akt signalling and osteoblast proliferation. Am J Pathol 169:1303–1311.

Hayashi M, Nimura K, Kashiwagi K, Harada T, Takaoka K, Kato H, Tamai K, Kaneda Y. 2007. Comparative roles of Twist-1 and Id1 in transcriptional regulation by BMP signalling. J Cell Sci 120:1350–1357.

Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz De Luna RI, Garcia C, Delgado M, Gonzales-Ramos AD, Kline EW. 1997. Mutations in Twist, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. Nat Genet 15:36–41.

Isenmann S, Arthur A, Zannettino AC, Turner JL, Shi S, Glackin CA, Gronthos S. 2009. TWIST family of basic helix-loop-Helix transcription factors mediate human mesenchymal stromal/stem cell growth and commitment. Stem Cells 27:2457–2468.

Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF. 2000. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 275:9645–9652.

Johnson D, Iseki S, Wilkie AO, Morriss-Kay GM. 2000. Expression patterns of Twist and Fgfr 1-2 and -3 in the developing mouse coronal suture suggest a key role for Twist in suture initiation and biogenesis. Mech Dev 91:341–345.

Kaabeche K, Lemonnier J, Le Mée S, Caverzasio J, Marie PJ. 2004. Cbl-mediated degradation of Lyn and Fyn induced by constitutive fibroblast growth factor receptor-2 activation supports osteoblast differentiation. J Biol Chem 279:36259–36267.

Kawamura N, Kugimiya F, Oshima Y, Ohba S, Ikeda T, Saito T, Shinoda Y, Kawasaki Y, Ogata N, Hoshi K, Akiyama T, Chen WS, Hay N, Tobe K, Kadowaki T, Azuma Y, Tanaka S, Nakamura K, Chung UI, Kawaguchi H. 2007. Akt1 in osteoblasts and osteoclasts controls bone remodeling. PLoS ONE 2:e1058.

Komaki M, Karakida T, Abe M, Oida S, Mimori K, Iwasaki K, Noguchi K, Oda S, Ishikawa I. 2007. Twist negatively regulates osteoblastic differentiation in human periodontal ligament cells. J Cell Biochem 100:303–314.

Lai CF, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV, Cheng SL. 2001. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 276:14443–14450.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16:307–310.

Lee MS, Lowe GN, Strong DD, Wergedal JE, Glackin CA. 1999. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J Cell Biochem 75:566–577.

Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, Stein JL, Stein GS. 2004. Regulatory controls for osteoblast growth and differentiation: Role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr 14:1–41.

Ling L, Murali S, Dombrowski C, Haupt LM, Stein GS, van Wijnen AJ, Nurcombe V, Cool SM. 2006. Sulfated glycosaminoglycans mediate the effects of FGF2 on the osteogenic potential of rat calvarial osteoprogenitor cells. J Cell Physiol 209:811–825.

Marie PJ. 2003. Fibroblast growth factor signalling controlling osteoblast differentiation. Gene 316:23–32.

Marie PJ. 2008. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys 473:98–105.

Marie PJ, Fromigué O. 2006. Osteogenic differentiation of human marrow-derived mesenchymal stem cells. Regen Med 1:539–548.

Marie PJ, Kaabeche K, Guenou H. 2008. Roles of FGFR2 and Twist in human craniosynostosis: Insights from genetic mutations in cranial osteoblasts. Front Oral Biol 12:144–159.

Miraoui H, Oudina K, Petite H, Tanimoto Y, Moriyama K, Marie PJ. 2009. Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signalling. J Biol Chem 284:4897–4904.

Oram KF, Gridley T. 2005. Mutations in snail family genes enhance craniosynostosis of Twist1 haplo-insufficient mice: Implications for Saethre-Chotzen syndrome. Genetics 170:971–974.

O'Rourke MP, Soo K, Behringer RR, Hui CC, Tam PP. 2002. Twist plays an essential role in FGF and SHH signal transduction during mouse limb development. Dev Biol 248:143–156.

Oshima A, Tanabe H, Yan T, Lowe GN, Glackin CA, Kudo A. 2002. A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, Twist. J Cell Biochem 86:792–804.

Oyajobi BO, Lomri A, Hott M, Marie PJ. 1999. Isolation and characterization of human clonogenic osteoblast progenitors immunoselected from fetal bone marrow stroma using STRO-1 monoclonal antibody. J Bone Miner Res 14: 351–361.

Pagès G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouysségur J. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci USA 90: 8319–8323.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Rice DP, Aberg T, Chan Y, Tang Z, Kettunen PJ, Pakarinen L, Maxson RE, Thesleff I. 2000. Integration of FGF and TWIST in calvarial bone and suture development. Development 127:1845–1855.

Rice R, Thesleff I, Rice DP. 2005. Regulation of Twist, Snail, and Id1 is conserved between the developing murine palate and tooth. Dev Dyn 234: 28–35.

Swaminathan G, Tsygankov AY. 2006. The Cbl family proteins: Ring leaders in regulation of cell signalling. J Cell Physiol 209:21–43.

Tang CH, Yang RS, Chen YF, Fu WM. 2007. Basic fibroblast growth factor stimulates fibronectin expression through phospholipase C gamma, protein kinase C alpha, c-Src, NF-kappaB, and p300 pathway in osteoblasts. J Cell Physiol 211:45–55.

Teplyuk NM, Haupt LM, Ling L, Dombrowski C, Mun FK, Nathan SS, Lian JB, Stein JL, Stein GS, Cool SM, van Wijnen AJ. 2009. The osteogenic transcription factor Runx2 regulates components of the fibroblast growth factor/proteoglycan signalling axis in osteoblasts. J Cell Biochem 107:144–154.

Thien CB, Langdon WY. 2001. Cbl: Many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2:294–307.

Yousfi M, Lasmoles F, Lomri A, Delannoy P, Marie PJ. 2001. Increased bone formation and decreased osteocalcin expression induced by reduced Twist dosage in Saethre-Chotzen syndrome. J Clin Invest 107:1153–1161.

Yousfi M, Lasmoles F, El Ghouzzi V, Marie PJ. 2002a. Twist haploinsufficiency in Saethre-Chotzen syndrome induces calvarial osteoblast apoptosis due to increased TNFalpha expression and caspase-2 activation. Hum Mol Genet 11:359–369.

Yousfi M, Lasmoles F, Marie PJ. 2002b. TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. Biochem Biophys Res Commun 297:641–644. Erratum in Biochem Biophys Res Commun 2003; 305:209.

Zhang Y, Hassan MQ, Li ZY, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2008. Intricate gene regulatory networks of helix-loop-helix (HLH) proteins support regulation of bone-tissue related genes during osteoblast differentiation. J Cell Biochem 105:487–496.